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A SIMPLE METHOD FOR PURIFICATION OF λ BACTERIOPHAGE ON WIDE-PORE GLASS

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13. ABSTRACT

Chromatography of wide-pore glass may be successfully used for purification of λ bacteriophage. A method for such purification was developed, and phage cultures were obtained with purification of the same order as in the best preparations reported. Optimal parameters of the chromatography process were found and the efficiency of the columns was evaluated.

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NOTICE

To obtain the preparation of a pure λ bacteriophage requires a precipitation with the help of hydroxyapatite and a fractionation with a hydroxyapatite column (2). The highly purified preparation gave for the ratio of the bacteriophage titer to the optical density at 260 mmk the value of

$3.6 \cdot 10^{11}$ $\frac{\text{plaque-forming units}}{\text{optical density units}}$. This study shows, how the same degree of purity can be achieved by filtering through a wide-pore glass (WPG) column. The principal way of purifying the bacteriophage by this method was described earlier (1).

Material and Methods

The basic material (WPG) used in this study was described earlier (1). λ bacteriophage represents the common wild phage received from the Jacob Laboratory (Pasteur Institute, Paris). It was titrated using the culture of E. coli C 600, obtained from the same source. Aminopeptide, from the Leningrad meat-packing plant, was used as the nutrition medium. Spectrophotometric measurements were made on the Spectrophotometer CF -- 4.

Results

The column packing material used was WPG, with pores measuring 1000 Å and 0.1 -- 0.3 mm fractions. The column with a cross section of 1.4 cm^2 was filled with powder to the height of 18 cm. The bacteriophage does not penetrate into the glass pores, since its size (length) is considerably larger than 1000 Å. All the impurities penetrate into the glass pores and are retained by them. The retention volume of the

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1. Bresler, S. E., Dobychin, D. P., and others, Molec. Biol. 3 No. 1, 1969, p. 29.
2. Thomas, C. A., Miyazawa, Y., J. Molec. Biol. 11, 1965, p. 223.

phage constitutes 15 ml. This is the total volume of the macroscopical pores between the grains. We measured this volume for DNK, separated from the E. coli, and obtained, as expected, the same value. The first experiments showed that the bacteriophage can to a considerable degree adsorb on WPG resulting in great losses. By using a buffer of appropriate ionic strength (0.1M tris pH 7.3 - 7.5 with 0.15 M NaCl), we reduced the adsorption of the phage and the connected with it losses to zero. The retention volume for the small molecules of inorganic salts is the geometric summation volume of all pores; equal to 24 ml. However, as it will be seen from the chromatograms, the eluted volume of impurities considerably exceeds the geometrical volume of pores. This means that many impurities adsorb on the glass even at the high ionic strength. And true enough, it was found that RNK and ribosomes actively adsorb to WPG. This favored the solution of the given problem.

The virus, subjected to purification from impurities, leaves the column first. Elution should be continued until the separation of all impurities is completed ($E = 0$ at $\lambda = 280$ ммк , $E = 0$ at $\lambda = 260$ ммк). After this the column should not be additionally regenerated, and it is ready for the purification of another portion. We cultivated the λ bacteriophage on the E. coli C 600 bacteria in an aminopeptide broth containing $5 - 7 \cdot 10^8$ plaque-forming units / ml. The cultivation on the cells was carried out for 20 min. at 37°. The phage was collected on soft agar disks during 12 - 18 hours. The phagasite was centrifuged for 30 min. at $5 - 6 \cdot 10^3$ rev. / min. The destroyed and the whole cells precipitated to the bottom of the tube. The top layer, containing the phage culture and a variety of high and low molecular components, was injected into the chromatographic column. The concentration of the phage in the centrifugate usually counted to 10^{10} plaque-forming units. It was found as the average of 3 titrations at 3 different dilutions. The degree of purity of the phage, in the resulting phagasite and after the chromatographic purification, was determined as the ratio of the phage titer to the optical density of the solution (E) at $\lambda = 260$ ммк. . The suspension volume of the phage, injected into the column, was usually 10 % of all the available volume of wide pores.

It is always advantageous to increase the concentration of the suspension. It can be easily concentrated 5 - 10 fold, if it is poured into a dialyzing sleeve with a 1 - 2 cm diameter and a room temperature air current is blown on it from the ventilator. The solution evaporates in 2 - 3 hours and losses in the yield of active phage are practically unnoticed. All the components become concentrated in relation to the initial and final volumes. Using this method, it is

possible to injeet a suspension with the concentration of $5 \cdot 10^{11}$ / ml. After the first chromatograph usually the total volume 10 - 15 ml of the bulk of the bacteriophage is collected. This consciously decreases the purity, but minimizes losses. After this the eluted volume is concentrated by evaporation to 0.5 - 1 ml and is subjected to a second chromatograph on a somewhat smaller column with a cross section of 0.8 cm^2 and a packing height of 15 cm of WPG. After the second chromatograph the separated phage, matches the purity of the standard (2).

Fig. 1 represents an experimental chromatogram, when the column is injected with 0.5 ml of the concentrated suspension with a titer of $5 \cdot 10^{11}$ / ml. In determining the relationship of the titer ($5 \cdot 10^{11}$/ ml) to the optical density ($E_{\lambda 260\ m\mu}$ = 162) of the removed fraction, the value turns out to be $3.1 \cdot 10^9 \frac{\text{plaque-forming units}}{\text{optical density units}}$; the contamination of the phage is in the order of 100 : 1 .

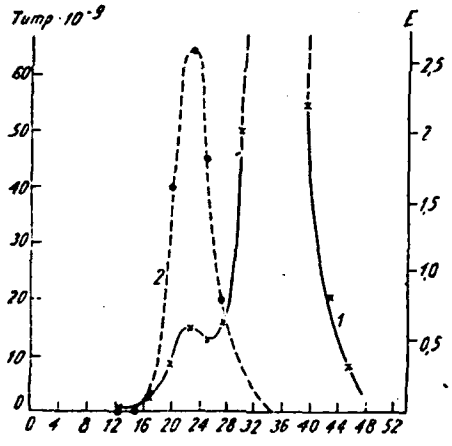

Fig. 1: Chromatogram of the concentrated λ phage suspension.

Parameters: the height of the column 18 cm, cross section 1.4 cm^2, injected volume 1 ml. Titer of the eluted volume $5 \cdot 10^{11}$ / ml. Rate of flow 0.4 ml / min. On the abscissa is the eluted volume (in ml); 1 is the extinction (E is the optical density at 260 mμ); 2 is the titer of the phage (number of plaque-forming units / ml).

From the chromatograph it is possible to determine the degree of purity within the peak. In the center of the peak the titer is $6 \cdot 10^{10}$ / ml and E = 0.6, as a result, the purity index is 10^{11} $\frac{\text{plaque-forming units}}{\text{optical density units}}$.

This means, that in the center of the peak the purity of the phage differs from the adjacent section by a factor of 3, and the overall effect of purification is 30 : 1. However, for the purpose of the second chromatograph the central fractions, as well as the adjacent fractions are collected to give a total volume of 15 ml. The contamination of the phage is obviously greater than in the center of the peak. After the concentration by evaporation, the purity index of the fraction is $1.7 \cdot 10^{11}$ $\frac{\text{plaque-forming units}}{\text{optical density units}}$.

0.5 ml of this suspension is injected into the column for the second chromatograph.

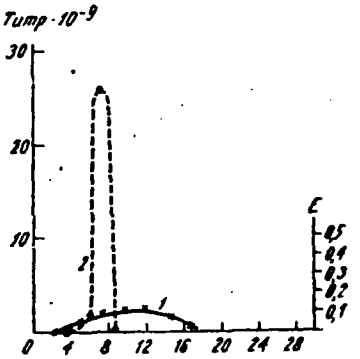

Fig. 2: The second chromatograph of λ phage.

Parameters: the height of the column 15 cm, cross section 1.4 cm^2, injected volume 0.5 ml. Titer of the phage in the eluted volume $6 \cdot 10^{10}$ / ml.
Rate of flow 0.4 ml / min.
1 and 2 are the same as in Fig. 1.

Fig. 2 shows the chromatogram of the second chromatograph. Practically speaking, the whole width of the peak encloses 2.4 ml. The titer of the peak (samples for the titers were taken out in 1.2 ml) is $2.5 \cdot 10^{11}$ / ml. In determining the

optical density of the solution, it is necessary to use a 2 cm cell to decrease the photometric measurement error. It can be seen from Fig. 2 that $E_{\lambda 260} \approx 0.08$ at the peak of the phage; from this the purity index is

$$3.2 \cdot 10^{11} \frac{\text{plaque-forming units}}{\text{optical density units}} .$$

The distinction of this value from the one found by Thomas is within the limits of the measuring error (10 %). Therefore, the two-stage purification is very effective.

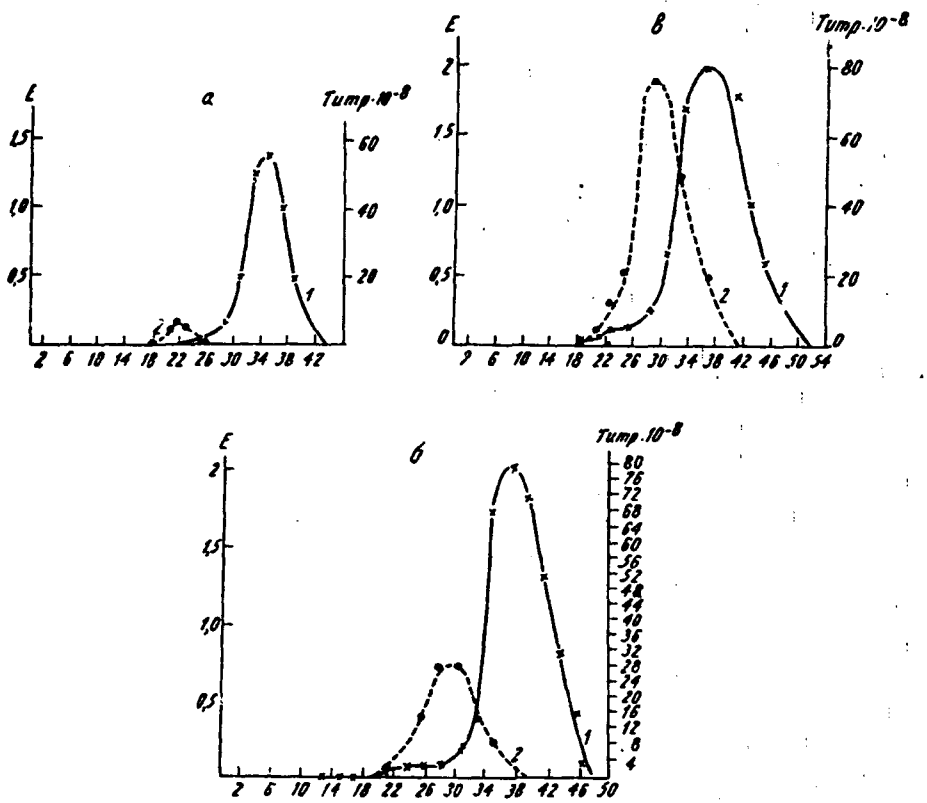

Fig. 3: The effect of the injected volume on the degree of purity of λ bacteriophage.

Parameters: the height of the column 22 cm, cross section 1.4 cm^2, injected volume 0.5 ml (а), 2.5 ml (б), 4 ml (в). Rate of flow 0.4 ml / min. 1 and 2 are the same as in Fig. 1.

Fig. 3, а, б, в, shows the effect of the injected volume on the width of the bacteriophage peaks. Within certain limits (up to the moment, when the injected volume equals 20 % of the available volume of wide-pores) a volume increase is permissible.

The effect of the flow rate on the degree of chromatographic purity is also studied. It is found that a three fold increase in the flow rate (0.4 - 1.2 ml · cm^2 / min.) does not affect the degree of purity of the phage.

Discussion

The obtained data shows that chromatography with WPG can be successfully applied to the purification of the λ bacteriophage.

The methods of this purification are worked out and the obtained phage culture has the purity of (ratio of the bacteriophage titer to the optical density)

$$3.2 \cdot 10^{11} \frac{\text{plaque-forming units}}{\text{optical density units}} \cdot$$

Since the changes in the flow rate, up to 1.2 ml ·cm^2 / min., do not affect the degree of the bacteriophage purity, then the elution time does not limit the effectiveness of the process.

The important factor in the determination of effectiveness, is the maximum volume that can be injected into the column. It should not exceed 20 % of the available volume of wide pores. Even for a small volume, injected into the column, the width of the peak is considerably large. Consequently, there are other factors besides the size of the injected volume, that affect the broadening of the peak.

It can be assumed that the spreading of the peaks is due to the adsorption of a small fraction of the phage to WPG. This is seen from the following: 1) the peak of the bacteriophage is considerably wider (3 to 4 fold), when the molecular separation is carried out with the (dirty) samples, rather than from the second chromatograph; it is possible, that it is related to the change in the glass surface after the adsorption of the fractions containing DNK and RNK; 2) the peak of the phage has the shape of the Lengmuire isothermal adsorption; 3) changes in the flow rate up to a factor of 3 do not effect the width of the peaks, therefore, the reason for the widening is the nondynamical (diffusion) processes.

The effectiveness of the column may be evaluated as the ratio of the maximum concentration of the phage (let us say, $5 \cdot 10^{11}$ / ml) to the maximum allowed volume (2.5 ml / cm^2 of glass). This gives 1.2 10^{12} plaque-forming units for an experimental column containing 15 g of WPG. With the increase of column's dimensions, the degree of retention will also increase proportionally to the increase in the volume and the weight of the sorbent. We assume, that the assembled data shows the promising aspects of the filtration method with WPG for the purification of viruses from impurities included in the cultured solutions.

In concluding we would like to thank D. P. Dobychin for consultations on the methods of acquiring WPG.

Bibliography

1. Bresler, S. E., Dobychin, D. P., and others, Molec. Biol. 3 No. 1, 1969, p. 29.

2. Thomas, C. A.. Miyazawa, Y., J. Molec. Biol. 11, 1965, p. 223.

Translator's note: mmk millimicron